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STUDIES ON THE HILL REACTION ACTIVITY OF SOLUBLE CHLOROPLAST EXTRACTS

UNPUBLISHED PRELIMINARY DATA

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Introduction

This study is concerned with the mechanisms and essential reactants of that part of the photosynthetic process in which oxygen is produced by photolysis of water. This is essentially the light absorbing reaction, which can be studied in vitro separately from the carbon dioxide fixing reactions. The photolysis of water and evolution of oxygen that occurs when chloroplasts or fragments of them are illuminated in the presence of a suitable electron acceptor is generally referred to as the Hill reaction. Attempts to reduce the Hill reaction system to a nonparticulate state have generally resulted in loss of activity. Progress toward this goal would be useful in defining the minimum components and conditions required for photolytic activity, as well as in determining the details of the mechanisms of energy transfer. Recent experiments along these lines in our laboratories led to establishment of this project to study chloroplast extracts.

Experimental Plan

The long range plan of study includes analytical investigations to identify and characterize the components of the photoactive complex, and to determine the function of each; examination of the role of proteins, especially enzymes, in the complex; studies on methods of preparing extracts of chloroplasts, and the stability of the preparations; determination of the participation and role of various cofactors; and application of spectroscopic and other physical methods to elucidation of the characteristics of the system.

Activity During the Period

Experimental investigation during this period included studies in two major areas: the effect of dimethylsulfoxide and caffeine on the Hill reaction activity of chloroplast preparations, and the development of preparative electrophoresis of chloroplast fractions in a carrier-free, high voltage unit.

Future Plan

The preparative electrophoresis studies will be continued, with the goal of separating active fractions and concentrating the effluent, for Hill reaction studies of these fractions. When the most active fraction is identified, it will be characterized in terms of proteins, pigments, plastoquinine, enzymes and cofactor requirements. Methods of stabilizing and improving Hill reaction activity will be extended.

Experimental

A. Chloroplast Preparations Containing Dimethyl Sulfoxide

Difficulty was encountered in the Sephadex chromatography of digitonin-solubilized chloroplast preparations. These studies were performed in a cold room, where the typical sucrose-phosphate buffer, described in Report No. 2, was highly viscous at 4°C. The sucrose is added to the buffer to increase the storage stability of the chloroplast preparations, especially those that are stored frozen.

Dimethyl sulfoxide (DMSO), which has been described as a useful nonaqueous solvent for many proteins, yields solutions without marked changes in the biological properties of the proteins [Rees and Singer, Arch. Biochem. Biophys. 63, 144 (1956); Ormsbee, Bell, and Lackman, J. Immunol. 88, 741 (1962); and technical information provided by Crown Zellerbach Corporation, Camas, Washington]. DMSO has unique solubility properties and is miscible in both polar and nonpolar solvents. Consequently, it was tested as a substitute for sucrose in the phosphate buffer. When a 0.4 M solution showed no inhibitory effect on the Hill reaction activity of a chloroplast preparation, the concentration of DMSO was increased to 10 percent wt/vol. The results of Hill reaction studies are summarized in Table I.

Table I

HILL REACTION ACTIVITY OF SOLUTIONS CONTAINING DMSO

Solvent System	Preparation	$Q_{O_2}^{Chl}$
0.4M DMSO	Chloroplasts	460
. . . . .	Fragments	383
Control	Chloroplasts	551
. . . . .	Fragments	439
10% DMSO	Chloroplasts	611
. . . . .	Fragments	520
Control	Chloroplasts	551
. . . . .	Fragments	611

DMSO may become a useful solubilizing agent. Within experimental error, the Hill reaction activity of solutions containing it was unchanged or slightly enhanced. Future studies should include its effect on the storage stability of chloroplast solutions kept at 4°C and at -40°C; its effectiveness in increasing digitonin solubility in aqueous solutions; and its potentiation or attenuation of energy transfer in the Hill reaction system.

B. Chloroplast Preparations Containing Caffeine

The recent paper by Fredericks and Jagendorf [Arch. Biochem. Biophys. 104, 39 (1964)] described both the preservation of Hill reaction activity and the marked stimulation of some photosynthetic systems by high molecular weight polymers, such as Carbowax and dextran. The earlier work of Clendenning, Brown, and Walldov was cited [Physiologia Plant. 9, 519 (1956)]. Clendenning advanced the thesis that the Carbowax compensated for the inactivation of chloroplasts by leaf tannins released into intimate mixture with the Hill reaction components. The Hill reaction rates were inversely related to the tannin content. Furthermore, it was impossible to regain Hill reaction activity once it was lost as a consequence of tannin-protein precipitation.

On another project, we have been using tannic acid as a quantitative protein precipitant. The proteins were recovered unchanged by applying the novel caffeine method of Mejbaum-Katzenellenbogen and co-workers [Nature 184, 1799 (1959); ibid., 192, 262 (1961); ibid., 193, 1288 (1962)]. Although spinach has a negligible tannin content, we tested the effect of 1 percent caffeine content on the Hill reaction activity of a typical spinach chloroplast preparation with the results shown in Table II.

Table II

HILL REACTION ACTIVITY OF SOLUTIONS CONTAINING CAFFEINE

Solvent System	Preparation	$Q \frac{Chl}{O_2}$
1% Caffeine	Chloroplasts	387
. . . . .	Fragments	352
Control	Chloroplasts	487
. . . . .	Fragments	851

Although there was a decrease in the Hill reaction activity of both whole chloroplasts and fragments, we do not consider 1 percent caffeine as a photosynthetic inhibitor. Stimulation of Hill reaction activity was reported by MacDowell [Plant Physiol. 24, 462 (1949)] for another class of alkaloids, e.g., brucine and strychnine. Caffeine should be tested as a Hill reaction stimulator, using a photosynthetic system high in tannin content. In addition, the enhancement of Hill reaction activity by strychnine should be confirmed. Information of this nature would be helpful in extending the biological half-life of the Hill reaction system.

C. Carrier-Free, Continuous High Voltage Electrophoresis

One of the simplest methods of separating chloroplast preparations into fractions is by sedimentation, which can be performed rapidly enough

to permit the study of Hill reaction activity in the fractions. Other biophysical procedures, however, are so time consuming that most of the activity is lost during fractionation. Electrophoresis is one method that has additional disadvantages. Until recently, the only preparative electrophoretic methods involved a solid support, such as a paper curtain. Unfortunately, the chloroplast pigment-protein complex has a marked affinity for cellulose. All of our attempts to study the mobility of the complex have been unsuccessful, because the sample remains at the point of application.

We have gained considerable experience in the use of high voltage preparative electrophoresis on the carrier-free instrument, the Elphor FF, designed by Hannig [Z. anal. Chem. 181, 244 (1961)]. In contrast to curtain electrophoresis, chloroplast preparations can be studied easily on a preparative scale and under high power conditions.

The Elphor FF has no carrier: it is essentially two air-cooled glass plates about 50 cm square, separated by a 0.5-mm space through which the electrolyte passes as a film. The anode was on the left side, and the instrument was run with the current electronically held constant. Forty-eight fractions divide the effluent at centimeter intervals, the fraction numbers increasing from left to right.

A Tris buffer (pH 7.5) was used at concentrations of from 0.15 M to 0.025 M. The lowest concentration gave a buffer with preferred ionic strength, which is less likely to produce uncoupling of the Hill reaction photophosphorylation, according to Good [Arch. Biochem. Biophys. 96, 653 (1962)]. In fact, the ideal buffer to overcome anionic uncoupling would be tris(hydroxymethyl)methylglycine or Tricine. Table III is a summary of the operating conditions of the Elphor FF, using dilute electrolytes.

The chloroplast fragments were obtained by extracting spinach leaves with Tris buffer of the same concentration used in the separating chamber of the Elphor FF. Fifty grams of deveined leaves were homogenized with 60 ml of Tris buffer in a Waring blendor for about one minute. The extract was squeezed through four layers of cheesecloth containing glass wool, and centrifuged at full speed (about 1000 X G) in a clinical centrifuge for one minute. The pellet of leaf tissue debris was discarded. The supernatant solution containing whole chloroplasts (P_1) and chloroplast fragments (P_2) was centrifuged at full speed in a clinical centrifuge for ten minutes. The pellet (P_1) was discarded, except where a digitonin extraction was made.

The supernatant liquid containing chloroplast fragments (P_2) was centrifuged for 15 minutes at 15,000 rpm (27,000 X G) in a Servall centrifuge. The high-speed pellets were resuspended in 15 ml of Tris buffer by homogenizing with a hand homogenizer. The solution was then centrifuged for ten minutes at full speed in a clinical centrifuge. The pellet was discarded and the suspension of P_2 was used in the Elphor FF.

Table III

OPERATING CONDITIONS FOR PREPARATIVE ELECTROPHORESIS

Expt. No.	Ma.	Volts	Dosing		Inlet Port	Buffer		Residence Time in Field (min)	Temp (°C)	Over-all Spread Between Fractions	TRIS Concentration (Molarity)
			Dial Setting	Rate (ml/hr)		Dial Setting	Rate (ml/hr)				
1	150	1950	1	3.0	38-39	2.5	153	41.7	4	19-29	0.05
2	150	1950	1	3.0	38-39	2.5	153	41.7	4	15-27	0.05
3	150	2000	2.0	4.8	38-39	2.5	153	41.7	4	16-35	0.05
4	150	1980	2.0	4.8	34-35	2.5	153	41.7	4	16-38	0.05
5	140	1850	4.0	8.6	34-35	2.5	153	41.7	4	15-37	0.025
6	140	1850	3.0	6.7	34-35	7.5	363	17.6	4	27-33	0.025
7	140	1850	3.0	6.7	34-35	2.5	153	41.7	4	19-33	0.025
8	0	0	3.0	6.7	34-35	2.5	153	41.7	4	33-37	0.025
9	140	1800	5.0	10.5	34-35	2.5	153	41.7	4	19-30	0.025
10	140	1850	4.0	8.6	34-35	2.5	153	41.7	4	17-29	0.025
11	70	1080	4.0	8.6	34-35	2.5	153	41.7	4	25-33	0.025
12	100	1450	4.0	8.6	34-35	2.5	153	41.7	4	23-34	0.025

In experiments involving digitonin extraction, the pellets (P_1) from slow speed centrifugation were resuspended in 20 ml Tris buffer containing 1 percent digitonin, allowed to stand for 15 minutes, and centrifuged for 10 minutes at 1000 X G. The pellets were discarded, but supernatant solution was then centrifuged in a Servall centrifuge for 15 minutes at 15,000 rpm (27,000 X G) to recover larger digitonin/chloroplast fragments (PD_2). The supernatant solution, containing very small digitonin/chloroplast fragments, was recentrifuged for one hour at 27,000 X G, and the pellets (PD_3) were resuspended in 5.0 ml of Tris buffer. The larger fragments (PD_2) from the 15-minute centrifugation were also resuspended in Tris buffer and prepared for the Elphor FF as described for the P_2 fragments.

Whole chloroplasts (P_1) agglutinated spontaneously in the separation chamber during electrophoresis. Since this phenomenon was rarely observed with chloroplast fragments (P_2), preparations of them were studied in detail.

In general, most of the proteins in the mixtures migrated toward the anode in the pH 7.5 buffer. The suspensions gave green patterns which broadened as much as 23 cm from their inlet points (Experiments 4 and 5). During the residence period in the chamber, the suspensions formed characteristic streaked patterns. Effluent was uniformly dispersed green suspension which gave typical fluorescence when irradiated with 365 mμ light.

With the exception of Experiments 2, 3, and 4, all of the experiments were performed with chloroplast fragments (P_2). Digitonin fraction PD_2 was studied in Experiment 2, and the smaller digitonin fragments PD_3 were used in Experiment 3. The corresponding supernatant solution SD_3 was also studied; it gave the very broad pattern under the conditions of Experiment 4.

Experiments 6 and 7 show the effect of varied buffer flow rate. At the high flow rate, the residence time in the electric field is shorter. Consequently, there is less resolution (spreading), but the effluent contains a higher concentration of chloroplast fragments. Experiments 7 and 8 can be compared as a measure of the effectiveness of electrophoresis. Experiment 8 (no power) showed a maximum spread of five fractions collected in a direct line below the inlet port, whereas Experiment 7 (38 volts/cm for 42 minutes) produced a broad pattern of 15 fractions skewed far enough toward the anode to be clear of any components with negligible mobility, had they existed. In Experiment 9, the increased rate of sample dosing broadened the pattern slightly.

An old sample of chloroplasts extracted with Tris and stored at 4° C for 11 days was studied in Experiments 10-12. In contrast, no satisfactory separation was obtained with chloroplast fractions prepared with the sucrose-phosphate buffer and stored at 4°C for 14 days prior to electrophoresis in the Elphor FF.

At a buffer flow rate of 153 ml/hr/48 tubes, the effluent is collected at 3.2 ml/hr/tube. A typical sample dosing rate of 6.7 ml/hr produces fractions containing chloroplast material that is four to eight times as dilute as the dosing solution. Consequently, some method of concentrating the effluent must be found before Hill reaction studies of oxygen evolution in the Warburg can be conducted precisely.

We are currently testing three methods of concentration: (1) direct addition of Sephadex; (2) dialysis against dry Aquacide; and (3) ultrafiltration. The photocatalytic dye reduction method might be more convenient for measuring Hill reaction activity and might eliminate the concentration step. Ultimately, we plan to characterize the components of an electrophoretically prepared system which shows Hill reaction activity.

Financial Status

During the fourth quarter, approximately 23 percent of the allocated funds were spent. Work will continue at about the same rate of effort during the fifth quarter.

Respectfully submitted,

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